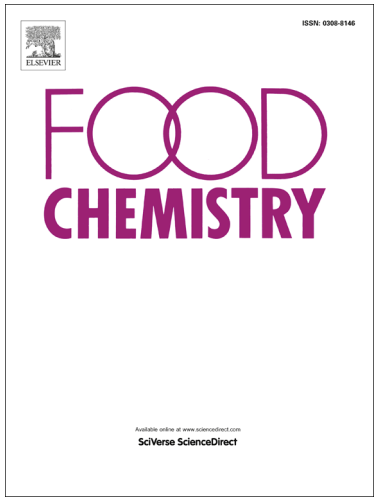
Floral nectar chitinase is a potential marker for monofloral honey botanical origin authentication



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Authentication honey origin by floral nectar chitinase activity

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Abstract

Honey，as a commercial product, is a target of adulteration through inappropriate production practices and deliberate mislabelling of botanical origin. Floral nectar protein could be a good marker for determining the source flowers of honey, especially monofloral honeys. Here, nectar and monofloral honey from *Eriobotrya japonica* Lindl. (loquat) were systematically compared, especially regarding proteomic and enzymatic activity. Using two-dimensional electrophoresis and mass spectrometry, only bee-originated proteins were detected in loquat honey. Xylosidase, thaumatin, and two kinds of chitinases were detected in loquat floral nectar, and their activity in loquat nectar and honey were quantified. Following gel electrophoresis, loquat honey had similar chitinase activity profiles to loquat nectar, but both were clearly distinguishable from *Camellia sinensis* nectar and *Brassica napus* honey. To our knowledge, this is the first examination of nectar-origin enzyme activity in honey. Zymography of chitinases is a potential marker for determining or authenticating the botanical origin of honeys.

Keywords： Authentication; Botanical origin; Chitinase; *Eriobotrya japonica* Lindl.; Floral nectar; Monofloral honey; Nectar protein

1. **Introduction**

Honey is a widely consumed natural food, produced by honeybees from the nectar of blossoms ("floral" or "blossom" honey), or the exudates of plant-sucking insects ("honeydew honey") (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). In this paper, the term "honey" refers to "floral honey" unless specifically stated otherwise. Honey is a complex product, containing about 200 substances, among which the largest portion other than water consists of sugars, mainly monosaccharides, fructose and glucose (Gallego-Pico, Garcinuno-Martinez, & Fernandez-Hernando, 2013). Other compounds present include minerals, proteins, amino acids, organic acids, lipids, pigments, phenolics, flavonoids, and vitamins; these are very important for honey characterisation and nutritive properties (da Silva et al., 2016).

Honey is classified as a premium product generally perceived as a high-quality and valued product because of its desirable flavour and taste. Consequently, honey has been a target of adulteration through inappropriate production practices and deliberate mislabelling of geographical and/or botanical origin. Honey can originate from single or multiple plant species, and this floral source largely determines its biochemical composition, flavour, and functional properties, including those that promote human health (Soares, Amaral, Oliveira, & Mafra, 2017a). Geographical location, climate, and honeybee species involved also have an effect on honey biochemical composition, as do to a lesser extent weather conditions, processing, manipulation, packaging and storage time (da Silva et al., 2016). In turn, the chemical components of honey can be analysed to determine its botanical origins, such as phenolics for heather (*Erica*) honey (Ferreres, Andrade, Gil, & Tomas-Barberan, 1996) and Australian monofloral *Eucalyptus* honey (Martos, Ferreres, Yao, D'Arcy, Caffin, & Tomas-Barberan, 2000);

hesperetin and methyl anthranilate for *Citrus* honey (Escriche, Kadar, Juan-Borrás, & Domenech, 2011); and carbohydrates for *Eucalyptus*, *Lythrum*, *Ammi visnaga*, and *Citrus* honeys (Terrab, Vega-Pérez, Díez, & Heredia, 2002). Analysis of free amino acids in honey is considered to be a good parameter for botanical and geographical origin identification; hence proline content is included in the European food laws for the quality parameters of honey (Soares et al., 2017a). However, adulteration of honey by adding special amino acids, e.g. proline, is easy to perform and low cost. In addition, the analysis of amino acids in honey requires expensive equipment, such as HPLC or an amino acid analyzer.

Honey proteins exist in minute quantities, and have been little studied (Soares et al., 2017a). The protein content and composition in honey has been used as a honey quality indicator in some countries, and for detection of adulteration (Bilikova & Simuth, 2010; Chua, Lee, & Chan, 2013; Dong, Xiao, Xian, & Wu, 2018; Won, Lee, Ko, Kim, & Rhee, 2008). However, most of the proteins so far identified in honey were of animal origin (Bilikova et al., 2010) and belonged to the family of major royal jelly proteins (Chua, Lee, & Chan, 2015; Di Girolamo, D'Amato, & Righetti, 2012). Apalbumin-1, the major protein of royal jelly, was reported to be especially prevalent and its concentration has been used to indicate honey adulteration with glucose syrups or by feeding bees with sucrose syrups (Bilikova et al., 2010; Simuth, Bilikova, Kovacova, Kuzmova, & Schroder, 2004). This and other bee-origin proteins may be useful to determine the bee species that produced a given honey (Chua et al., 2013; Ramon-Sierra, Ruiz-Ruiz, & Ortiz-Vazquez, 2015; Won et al., 2008), but not to determine its botanical origin (Bilikova et al., 2010; Simuth et al., 2004). Nonetheless, honey protein mass spectra profiles can be used to authenticate the purity and geographical origin of honey in commercial trade (Wang, Kliks, Qu, Jun, Shi, & Li, 2009).

Honey proteins arising from pollen and/or as a result of the enzymatic reaction between bee saliva and plant pollen might also be useful markers for distinguishing between types of honey produced by the same bee species (Baroni, Chiabrando, Costa, & Wunderlin, 2002). Furthermore, a sensitive enzyme-linked immunosorbent assay (ELISA) method was developed to identify pollen in honey using two sunflower pollen specific proteins as antigens (Baroni, Chiabrando, Costa, Fagúndez, & Wunderlin, 2004). This immunoblot method opens an interesting field for the assessment of honey floral origin but it requires the development of new antibodies from different plants which is costly and time consuming.

A comparison of honeys using a gel-based proteomic approach identified only one protein of plant origin (a glyceraldehyde-3-phosphate dehydrogenase from *Mesembryanthemum crystallinum*), providing limited utility for determining botanical origin (Di Girolamo et al., 2012). Therefore, the predominance of bee-origin proteins in honey may impede the detection of less abundant plant-origin proteins via regular gel-based approaches, and alternative methods may be needed. Using gel-free based analysis with high definition mass spectrometry, *Brassica napus* pollen proteins were identified in honey and deemed to be important for the nutritional value of plant pollen-enriched honey (Borutinskaite et al., 2017). However, very few studies have examined the existence of nectar proteins in honey, or explored their utility for honey botanical origin authentication.

It has long been known that floral nectar contains proteins, generally less than 100 μg mL^{-1} (Roy, Schmitt, Thomas, & Carter, 2017). Few specific proteins are present, but they are usually enzymes, and species specific. Most of these are classified into glycosyl hydrolase or pathogenesis-related proteins, such as chitinase, glucanase, xylosidase, galactosidase, etc. (Heil, 2011; Park & Thornburg, 2009; Roy et al., 2017). Honey production involves regurgitation,

enzymatic activity, and water evaporation but not digestion. Proteases have occasionally been detected in honey (da Silva et al., 2016); moreover the environment within honey is highly concentrated and usually acidic, both of which would restrict the activity of proteases and other enzymes. Therefore, floral nectar proteins might survive the process, become more concentrated, and retain enzymatic activity in mature honey, though this has yet to be tested. If so, such activity might permit their detection and identification, even in spite of larger quantities of bee-origin protein being present. This offers a potential new means of determining the botanical origin of honey.

The loquat (*Eriobotrya japonica* Lindl.; Rosaceae) is a subtropical evergreen fruit tree indigenous to subtropical regions of China, but cultivated worldwide for its edible fruits, which are eaten fresh or processed as jam and other products (Lin, Sharpe, & Janick, 2010). Loquat leaves and fruits are also used as a traditional medicine for the treatment of coughs, skin diseases, and diabetes (Lin et al., 2010). Loquat is also a well-known honey plant, flowering from around November to January, with honeybees as the major pollinator. Loquat honey is well known for its unique flavour and effect of cough relief (Lin et al., 2010), valued therefore at four times the price of rapeseed honey (Zha H.G., Feburary 2018, personal observation). Because of this, loquat honey is also a target for adulteration by supplementing bee diets with sucrose, or mixing with other cheap honey. Moreover, loquat honey is claimed to be a monofloral honey, which would be invalidated if it is mixed with other nectar sources (Caballero & Fernández, 2003). For example, *Camellia sinensis* blooms at the same time and provides ample nectar, and where *C. sinensis* grows close by (Zha H.G., personal observation), there is as yet no way to prove that loquat honey is monofloral.

In this study, we used gel-based proteomic techniques to compare the proteomes of loquat nectar and honey, aiming to identify loquat floral nectar proteins from loquat honey. We also compared the nectar protein enzymatic activities in the floral nectar and honey samples to test whether the nectar-originated enzymatic activity could be used as a marker for honey botanical origin authentication.

2. **Materials and methods**

2.1 Floral nectar and honey samples

Each loquat (*Eriobotrya japonica*) inflorescence comprised a number of 10-25 flowering units, including very young buds to fully opened flowers. *E. japonica* secretes 5~20 μL of nectar per flower. Raw floral nectar was collected from loquat flowers with pipettes and autoclaved tips in the mornings between December 2016 and January 2017 at SheXian county, Anhui province, China (118º 32'47''E; 29º 49'15''N). On each of four days, all floral nectar samples collected were pooled as an individual samples (5-10 mL each), making four nectar samples in total. These were then filtered through 0.22 μm membrane filters (Merck Millipore) to remove pollen granules and dirt from the pooled nectar samples. Three raw floral nectar samples from nearby tea plants (*Camellia sinensis*) at the same sampling place, were also collected and treated as for loquat nectar and used as a reference sample in this study. Three loquat honey samples (1 kg each) were purchased from two trustworthy local beekeepers, whose hives are within 200 m of the nectar sampling sites, in January 2017. A single sample of monofloral honey from *Brassica napus* was collected from beekeepers in the same county in May 2017 and used as a reference. All floral nectar and honey samples were stored at -80 ℃ prior to use.

2.2 Physicochemical analysis

Total sugar concentration of pooled nectar samples was estimated as the Brix value, obtained using a low-volume hand-held refractometer (Eclipse, Bellingham & Stanley, Tunbridge Wells, UK). Honey moisture content was determined from the refractive index at 20 ℃ using a honey refractometer (HHR-2N, Atago, Japan). The pH of fresh nectar and diluted honey samples (10% solution with distilled water) was measured using a pH meter (Model FiveGo F2, Mettler Toledo)

with an InLab Micro Probe (Mettler Toledo). The total phenolics content of nectar and honey samples was measured using the Folin–Ciocalteu method with Gallic acid as a standard (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). The total phenolics content was expressed in µg of Gallic acid equivalents (GAE) mL^{-1} of nectar or µg of GAE g^{-1} of honey. The determination of sugars in nectar and honey samples were performed with an EClassical 3100 high-performance liquid chromatograph (Elite, Dalian, China) equipped with a refractive index detector (RI-201H, Shodex, Japan). A carbohydrate column (SC1011, Shodex, Japan) was used for the separation and degassed water was used as an eluent for analysis at a flow rate of 0.8 mL min^{-1} at 85 ℃. The protein content in the pooled nectar and honey samples was determined according to Bradford (1976) method, using bovine serum albumin as a standard. Reported values are the average of triplicate experiments.

2.3 Honey and floral nectar protein extraction and 1D/2D gel electrophoresis

Honey samples were dialysed against distilled water for 24 hours at 4 ºC to remove sugars and other metabolites until the sugar concentration was lower than 10 Brixº. Dialysed honey and fresh nectar protein was concentrated to reach final concentration of 1mg mL^{-1} by ultra-centrifugal filtering with Amicon Ultra filter (cut-off 10000 Da; Merck Millipore).

For 1D electrophoresis, ten micrograms of concentrated loquat nectar and honey proteins were heated at 65 ºC for 10 minutes in SDS sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue) under reducing conditions (with 0.1 M dithiothreitol [DTT] in sample buffer). Those were then loaded into 12% (w/v) self-poured sodium dodecyl sulfate polyacrylamide gel, and separated using electrophoresis according to Laemmli (1970). Prestained protein ladder (ranging from 15,000 to 150,000 Da; Sangon Biotech, ShangHai, China) were used as a marker. After electrophoresis, one gel was stained for protein with

Coomassie Brilliant Blue (CBB) G250, while another was stained for glycoprotein with the Pierce™ Glycoprotein Staining Kit of the periodic acid–Schiff method (Thermo Scientific) according to the manufacturer's instructions. Two-dimensional gel electrophoresis was performed using a ReadyStrip immobilized pH gradient (IPG) Strip and PROTEAN IEF Cell (Bio-Rad). Briefly, for loquat honey and nectar proteins, 7 cm linear pH 3–10 IPG strips were rehydrated for 12h with 125 µL of rehydration solution (8 M urea, 65 mM DTT, 4 % [w/v] CHAPS, and 0.2 % [w/v] Biolyte 3/10 Ampholyte [Bio-Rad]) containing 20 µg of total proteins. Isoelectric focusing (IEF) was run at 20 °C according to the 2-DE manual of Bio-Rad with a 7-cm strip, pH gradient from 3 to 10, for a total of 20 kVh. After IEF, the IPG Strips were equilibrated with an equilibration buffer (0.375 M Tris-HCl, 6 M urea, pH 8.8, 20% [v/w] glycerol, and 2% [w/w] SDS) with 2% DTT for 10 min for the first equilibration step and then for 15 min in the equilibration buffer containing 135 mM iodoacetamide instead of DTT. For SDS-PAGE, the equilibrated IPG strips were transferred onto 12% polyacrylamide gels by use of a Mini-PROTEAN Tetra gel electrophoresis system (Bio-Rad), then electrophoresis was run at 180 V constant for one hour, following the instructions of the manufacturer. The proteins on 2-DE gels were visualized by CBB G250 staining. Samples were run in triplicate.

2.4 In-gel digestion and mass spectrometry analysis

The protein spots were excised from 2-DE gels and subjected to in-gel digestion performed with trypsin as the protease. The protein samples were analyzed by tandem mass spectrometry (MS/MS) using a 5800 matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (AB SCIEX). The MS/MS spectra were extracted and analyzed with ProteinPilot 4.5 software (AB SCIEX) with a Mascot search program (www.matrixscience.com/) and searched against Swiss-Prot and NCBInr databases (considering all entries for proteins from

honey, but only plant entries for nectar proteins). Proteins with a MASCOT score higher than 50 were considered to have been clearly identified. The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org/submission/index.html) via the PRIDE partner repository (Vizcaino et al., 2016) with the data set identifier PXD010083.

2.5 Enzyme assays

Chitinase activity of loquat honey and floral nectar was measured by a fluorimetric chitinase assay kit (Sigma-Aldrich) following the manufacturer's instructions. In brief, for each individual experiment, 10 µl of nectar or 10 mg honey were incubated with 90 µl substrate working solution (4-methylumbelliferyl β-D-N,N',N''-triacetylchitotriose; 0.5 mg mL^{-1}) at 30 ℃ for 30 min. The reaction was stopped with 100 µl 0.8 M sodium carbonate solution. The amount of 4-methylumbelliferone (4-MU) released was then measured on a SpectraMax i3x Microplate Reader (Molecular Devices Inc.) at excitation and emission wavelengths of 360 nm and 450 nm, respectively. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol 4-MU from the substrate per min at pH 5.0 and 30 °C, which approximated the pH of raw loquat nectar.

Loquat nectar and honey β-D-xylosidase activity was measured according to Nepi, Bini, Bianchi, Puglia, Abate, and Cai (2011) with minor modifications. The method is based on the enzymatic reaction between xylosidase and 4-nitrophenyl β-d-xylopyranoside (pNpX) which generates p-nitrophenol whose concentration is quantified spectrophotometrically at 410 nm. A 10 µL aliquot of loquat nectar or 10 mg honey sample was mixed with 15 µL of citrate buffer (100 mM at pH 5.0) and 25 µL of 10 mM *p*NpX (Sigma) solution. The reaction was carried out at 30 °C for 10 min and then stopped by the addition of 150 µL of 0.2 M borate buffer (pH 9.8) to the assay mixture. A reference blank was obtained by substituting the nectar or honey with an

equivalent volume of 20% sucrose solution. The experiment was performed in triplicate. One unit of activity is defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min, as measured by absorbance at 410 nm.

Because proteases could degrade and inactivate other enzymes, the protease activity of loquat nectar and honey was measured spectrometrically using casein as the substrate, according to Cupp-Enyard (2008).

Protein-free loquat nectar or honey was used as a reference for these enzymatic activity assays. Proteins were removed from loquat nectar and honey (diluted four times with distilled water) using ultracentrifugal filtering with Amicon Ultra centrifugal filters (cut-off 3 kDa; Merck Millipore).

2.6 In-gel chitinolytic activity staining

Profiling of loquat honey and floral nectar chitinolytic activity in gel after SDS-PAGE was performed according to Trudel and Asselin (1989) and Zha, Milne, Zhou, Chen, and Sun (2016) with minor modifications. Briefly, honey was dialysed to reduce viscosity, and brought to 10 Brix°. Then fresh nectar (20 μL per well) and dialysed honey from both loquat and tea plant were loaded in gels without boiling beforehand. After SDS-PAGE, gels were incubated at 28 °C for 3 h in 50 mM sodium acetate buffer (pH 5.0) containing 0.5% Triton–X 100 (v/v), to promote chitinase activity against glycol chitin which was prepared from glycol chitosan (Sigma) and remove SDS. The gels were then stained with 0.01% Calcofluor white M2R (Sigma) in 50 mM Tris–HCl (pH 8.9) and chitinase activity determined using a UV transilluminator. The clear lytic zones of chitinase isoforms were visualized as dark bands against a fluorescent background under the UV transilluminator, and then photographed.

2.7 Melissopalynological analysis

The pollen analysis was performed according to the harmonized method proposed by von der Ohe, Persano Oddo, Piana, Morlot, and Martin (2004). 10 g of loquat honey was dissolved in 20 mL distilled water and centrifuged at 3500g for 10 min at 20 °C. The obtained sediment was then re-dissolved in 20 mL distilled water to completely remove the remaining sugar crystals and centrifuged for a further 10 min. Pollen sediment was mounted in glycerine jelly and checked using a Motic BA410E light microscope (Motic China Group Co., Ltd., Xiamen, China) at a magnification of 400×. Four hundred grains of pollen were identified for the loquat honey sample. Pollen identification was based on the reference collection of *E. japonica* and *Camellia sinensis* pollen from the Laboratory of Plant Ecophysiology, Huangshan University.

3. Results

3.1 *Eriobotrya japonica* (loquat) floral nectar and honey basic traits

E. japonica nectar was acidic, having a pH value of 5.1 and contained a mean total sugar concentration of 21.0 Brix^{o}. Nectar was pooled for all analyses. In pooled raw loquat nectar, the mean concentration of total phenolics was 27.2 μg GAE mL^{-1}, whereas the mean total protein content was 12.2 μg mL^{-1}.

Loquat honey samples collected from local bee keepers were white, with water content of 17.5% which was lower than the cut-off value at ≤ 20% (da Silva et al., 2016). The mean concentration of total phenolics in loquat honey samples was 177.3 μg GAE g^{-1} which is lower than that in some other reported honey samples (Meda et al., 2005; Soares, Pinto, Rodrigues, Alves, & Oliveira, 2017). This might be caused by the relatively low total phenolics level in the source, i.e. loquat nectar. Loquat honey in the study was more acidic than loquat nectar with pH values of 3.6 and 5.1, respectively. The mean total protein content in loquat honey samples was 520 μg g^{-1} which is close to that reported for reported Citrus and Eucalyptus honeys (Azeredo, Azeredo, de Souza, & Dutra, 2003).

3.2 Sugars in loquat floral nectar and honey

According to HPLC, the ratio of sucrose, glucose and fructose in loquat nectar is 9.6 : 1 : 1 (Fig. 1), whereas that in loquat honey is 1 : 2.9 : 3.1. This indicates that sucrose hydrolysis via invertase or other sucrases might play roles in the ripening of loquat honey. Comparing with other reported honey samples, the high sucrose content in loquat honey might be caused by the high sucrose in the source nectar and/or low invertase activity in the cold season when the honey was produced (Soares et al., 2017a). Sucrose is often used to adulterate honeys, and hence

sucrose content has been used as a marker to detect honey adulteration; however the above result suggests that this method might not work due to sucrose hydrolysis following honey adulteration or during honey production.

3.3 Proteins in loquat floral nectar and honey

Both concentrated loquat honey and nectar proteins were separated using SDS-PAGE and visualized by CBB G250 (Fig. 2A). On the gel, loquat honey proteins exhibited a series of zones estimated spanning the 5 to 100 kDa interval, the most abundant one was estimated to centre at 55 kDa. The profile was similar as other monofloral honey proteins, such as from chestnut, sunflower, orange, acacia and rapeseed (Borutinskaite, et al., 2017; Di Girolamo et al., 2012). However, under the same conditions, loquat nectar had a different protein profile from honey, yielding 10 bands ranging from 18 to 100 kDa but none of the proteins predominated (Fig. 2a). Loquat honey was rich in glycoproteins, but although some glycoproteins were also detected in loquat nectar, they showed very different banding profiles (Fig. 2b). Glycoproteins with apparent MW from 40 to 100kDa from loquat honey were not detected in loquat nectar, indicating that loquat honey glycoproteins were not of plant origin.

Two-dimensional electrophoresis revealed a different protein profile for loquat nectar and honey (Fig. 3). Most loquat honey proteins ranged in molecular mass estimated from 5 to 100 kDa, consistent with the patterns observed in 1D gel (Fig. 2). However, a much smaller number of nectar proteins than expected were detected by 2-DE, most of which ranged in molecular mass from 15 to 25 kDa. From the loquat nectar 2-DE gel, 7 visible spots were successfully analysed by mass spectrometry, representing 5 proteins: class III chitinase, class IV chitinase-1, class IV chitinase-2, xylosidase and thaumatin (Fig. 3a； Supplemental material Table S1). From loquat honey 2-DE gel, 30 spots were identified, but these only represented 4 proteins, all with bee

origins: three major royal jelly proteins and an uncharacterized protein (Fig. 3b； Supplemental material Table S2). Among these, major royal jelly protein-1 from *Apis mellifera* was predominant, accounting for 19 spots from the 2-DE gel. This is consistent with previous findings that this protein is the dominant proteinaceous component in honey and prone to be degraded during the honey ripening and storage (Borutinskaite, et al., 2017; Di Girolamo et al., 2012). Failure of the 2-DE gel approach to detect floral proteins in honey is consistent with suggestions that these might be digested by honeybees, or degraded during honey ripening (Borutinskaite, et al., 2017; Di Girolamo et al., 2012). However, another possibility is that the concentration of floral nectar proteins in honey might be at an extremely low level, and/or obscured by high-abundant bee-originated proteins in honey when using routine proteomics approaches. We also employed a gel-free proteomics approach (LC–MS/MS), but this method consistently failed to detect any plant originated proteins such as chitinase in the honey (data not shown).

3.4 Chitinase and xylosidase activity quantified in loquat honey and nectar

Chitinase and xylosidase proteins were identified in the loquat floral nectar using 2-DE and mass spectrometry, and if present and intact in honey, these should be detectable by their enzyme activity. Using 4-MU-triacetylchitotriose as the substrate, the universal chitinase activities in loquat nectar and honey were quantified to be 7.54 and 0.11 U mg^{-1} protein, respectively, whereas no activity was detected in protein-free loquat nectar or honey. Hence chitinolytic activity in honey is solely due to chitinase but occurs at a greatly reduced level relative to nectar. The chitinase activity in the reference samples, i.e. *Camellia sinensis* floral nectar and rapeseed honey, had mean values of 1.16 and 2.44 U mg^{-1} protein, respectively.

The xylosidase activities in loquat nectar and honey were determined to be 1.81 and 0.14 U mg^{-1} protein, respectively, using 4-nitrophenyl β-d-xylopyranoside as the substrate. No protease activity was detected in loquat nectar or honey using casein as substrate.

3.5 Loquat nectar and honey chitinase zymography

Chitinase zymograms revealed clear similarity between loquat honey and nectar (Fig. 4, lane 2 and 3). Both loquat honey and nectar samples showed two major bands and one faint band with the same mobility, consistent with most of the chitinases in honey being from nectar. Loquat honey had one extra faint band with chitinolytic activity and a molecular weight of 21 kDa, which was not detected in loquat nectar. This could be of bee origin, or a truncated loquat origin chitinase. *C. sinensis* floral nectar had a one-banded phenotype, whereas rapeseed honey had a 5-banded phenotype; none of these bands had similar mobility to any within the loquat samples (Fig. 4, lane 1 and 4).

3.6 Palynological characteristics of loquat honey

Monofloral honey generally refers to the presence of a single pollen type in quantities higher than 45% of the total pollen content (von der Ohe et al., 2004). Microscopic examination of pollen (melissopalynology) showed that loquat pollen was the predominant type of pollen in loquat honey sample (Supplemental material Fig. S1), representing >95% of pollen present. Of the remaining 5%, some could not be identified, but the distinctive pollen grains of *Camellia sinensis* were not found, despite the species being present close to the hives, and in flower at the same time as loquat.

4. Discussion.

4.1 Nectar-origin enzymes activity was detected from honey

Plant-origin proteins are a minute component of the honey proteome, and have therefore been difficult to detect using routine proteomic techniques, due to the masking presence of more abundant proteins of animal origin. This has so far prevented the use of proteins in determining the botanical origin of honey. In this study, we demonstrated that the loquat floral nectar proteome consisted of xylosidase, thaumatin, class III and class IV chitinases, but none were detectable from loquat honey using proteomic approaches. The loquat honey proteome mainly comprised major royal jelly proteins from bees.

Most of the proteins so far identified from nectar have been enzymes, e.g. chitinase, glucanase, galactosidase, xylosidase and others (Heil, 2011; Park et al., 2009; Roy et al., 2017). However, few of these floral nectar enzyme activities had been analyzed in honey before. Conversely, while many honey proteins are also enzymes (e.g. invertase, diastase, glucose oxidase, catalase, and acid phosphatase), none of these are known from floral nectar, and they are hence presumed to have been added by the bees (da Silva et al., 2016). The predominant proteins in honey are major royal jelly proteins, with no known enzymatic activities. Therefore, we tested the idea that enzymatic activity might permit detection of floral proteins in honey, where other methods do not. In addition, we detected no proteolytic activity in nectar or honey samples, indicating that nectar origin proteins might remain intact for a long time in honey. In this study, we successfully detected and compared the xylosidase and chitinase activities in honey and the corresponding nectar samples.

4.2 Plant chitinases in nectar and honey

Chitinase plays a direct role in plant defence by attacking the cell walls of bacteria, algae and fungi, and the exoskeletons of arthropods, within which chitin is a major component. It is hence deemed to be a pathogenesis-related protein implicated in defence mechanisms for reproductive organs, occurring in pollination drops, floral nectar and extrafloral nectar (Heil, 2011; Ma, Milne, Zhou, Fang, & Zha, 2017; Zha et al., 2016). However, there is as yet no empirical evidence showing that chitinases in nectar harms insect pollinators, such as honey bees (Zha et al., 2016). Plant chitinases also function in flower development, leaf senescence, embryogenesis, seed development and post-translation modification of glycoproteins (Grover, 2012).

Chitinases have now been repeatedly identified in the floral nectar of different plant species, including important honey plants (Heil, 2011; Ma et al., 2017; Zha et al., 2016). Hence it is not surprising to detect chitinase activity in honey as in this study; indeed, it may be common in honey but have been previously overlooked. Insects including honeybees do produce chitinases and chitinase-like proteins (Arakane & Muthukrishnan, 2010), but these can be distinguished by their sequence and traits of enzymatic activity, and no chitinase of bee origin was detected by 2DE and mass spectrometry in the current study. Instead, the similarity of chitinase zymography pattern between loquat honey and nectar samples indicates that most or all of the chitinases in loquat honey are of plant origin. Because the chitinases in honey are usually of plant origin, and they differ between species, it could be possible to use chitinase as a marker for honey botanical origin authentication.

4.3 Nectar-originated chitinases meet the requirements to be used for honey botanical origin authentication

Chitinases are well-studied enzymes, all seven classes of which have been characterized in nectar (Zha et al., 2016). Moreover, multiple classes may occur in nectar of a single species, e.g. class I plus II chitinases in *Nicotiana attenuata* and *Petunia* (Hillwig, Kanobe, Thornburg, & MacIntosh, 2011; Seo et al., 2013), and Class II plus class III chitinases in *Rhododendron irroratum* (Zha et al., 2016). Different classes of chitinases can differ greatly in sequence, structure, catalytic mechanisms and mobility during electrophoresis (Grover, 2012), and even within chitinase classes, variation may occur in enzyme molecular weight, and hence mobility during electrophoresis (Zha et al., 2016). Therefore, many or all nectar-producing species will have a multi-band chitinase zymography signature, and signatures may differ even between closely related species (Zha et al., 2016). In the case of loquat nectar, two classes of chitinase were identified; furthermore loquat honey and nectar had very similar chitinase zymograms but both were completely different from those of rapeseed honey and *Camellia sinensis* nectar.

Most chitinases are very stable proteins (Grover, 2012), some of which do not lose activity after heating at 65℃ for ten minutes (Ma et al., 2017; Zha et al., 2016). Therefore, chitinase activity is likely to survive the transformation from nectar to honey, as supported by similar chitinase zymographs between loquat nectar and honey. Furthermore, chitinase activity in loquat honey sample is barely reduced after one year's storage at 4 °C in our lab (data not shown), implying that such activity also could be detected in honey after transportation, storage in warm environments, and even some industrial processes such as filtration and centrifugation. Approximate dehydration environment is another important positive factor for preserving enzyme activity for a long time in honey. Chitinases are also resistant to such detergents as high concentration sodium dodecyl sulfate (SDS), making it possible to use SDS-PAGE (which has higher resolution than native-PAGE) for chitinase separation.

Other nectar enzyme groups, such as xylosidase, show much less class variation and polymorphism than chitinase. Moreover, both qualifying and quantifying of chitinase activity are relatively straightforward and crucially do not require expensive equipment such as a mass spectrometer. Therefore, we suggest that chitinase zymography could be a suitable tool for honey botanical authentication, although more comparative chitinase zymography work between nectar and honey samples, and across species, will be necessary. In this study, the chitinase zymogram of loquat honey matched that of loquat nectar alone, rather than being additive between loquat and, for example, the locally abundant *Camellia sinensis*. Hence, based on the chitinase zymography, we found no evidence that nectar of *C. sinensis*, or any species other than loquat, made up part of the source for the loquat honey examined. This may be because honeybees prefer *E. japonica* nectar to *C. sinensis* nectar for reasons unknown. During the time when we collected loquat floral nectar (four full mornings in the early winter of 2017), we noticed that most of the honeybees were concentrated on loquat flowers, whereas few visited the *C. sinensis* flowers blooming nearby. In addition, even though *C. sinensis* is the main cash crop in south China (including the region we investigated), and the plant secretes ample floral nectar, we found no evidence that this species contributes to honey production. . Consistent with this, the predominant pollen detected in loquat honey came from loquat, so all evidence indicates that loquat honey is indeed monofloral.

Even though chitinase is relatively stable, its activity in honey will still fade along with the storage time, and reduced by light, heat, and other energy sources (e.g. microwaves). Therefore, further investigation is needed to determine whether the content and activity of chitinase in honey could be used as a quality parameter following processing and/or storage. Regarding deliberate adulteration of honey, e.g. to pass off cheap honey as an expensive kind, it is relatively

cheap and easy to fake a honey signature by adding pollen and/or amino acids, but particular chitinases would be extremely difficult to obtain in the necessary quantities, because floral nectar is the only available source. Hence a chitinase zymogram signature of honey might be nearly impossible to fake.

5 Conclusions

Plant floral nectar proteins are very minor components in the honey proteome and are hard to detect using routine proteomic techniques. However, the activities of floral nectar proteins can be quantified and phenotyped in honey and used as potential markers for authentication of honey botanical origin. Chitinases have been detected from many plant species' nectar, are stable, and can remain active in honey after regular honey production processes. In this study, we demonstrated that the zymography of chitinases is polymorphic, and can distinguish loquat honey and nectar from rapeseed honey and *Camellia sinensis* nectar.

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Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical statement

Our work complies with the ethical rules applicable for this journal.

Author Contributions

Conceived and designed the experiments: HGZ, YQS, JYF. Performed the experiments: YQS, XLM, HXZ. Analyzed the data: HGZ, RIM. Wrote the paper: HGZ, RIM.

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Figure legends

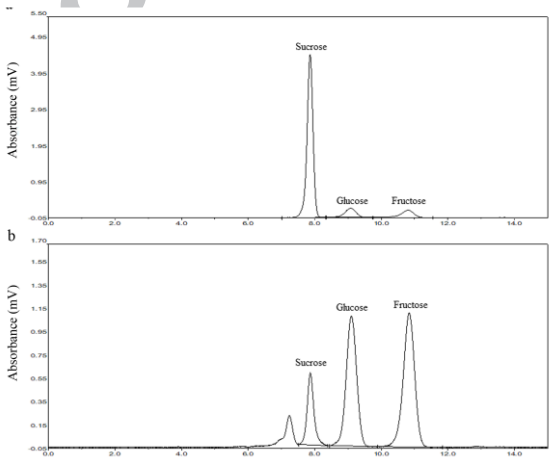
Fig. 1 HPLC chromatogram of loquat floral nectar and honey. a, loquat floral nectar; b, loquat honey.

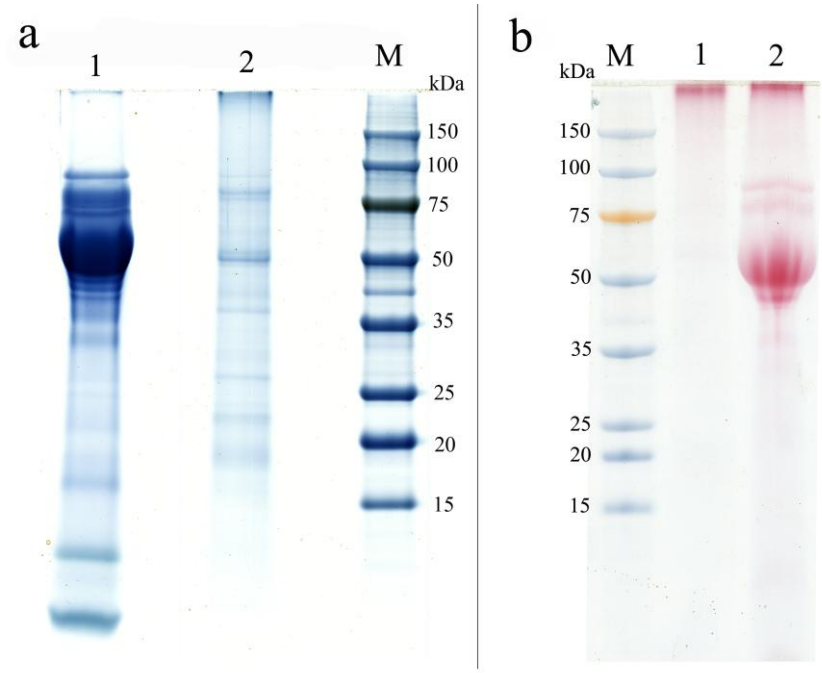
Fig. 2 SDS-PAGE of loquat nectar and honey proteins treated with CBB (left) and glycoprotein (right) staining. a, CBB staining. Lane 1 contains loquat honey proteins; lane 2 contains nectar proteins under reducing conditions. b, Glycoprotein staining. Lane 1 contains loquat nectar proteins; lane 2 contains loquat honey proteins. Lane M shows different ranges of reference proteins with the molecular masses at the standards indicated.

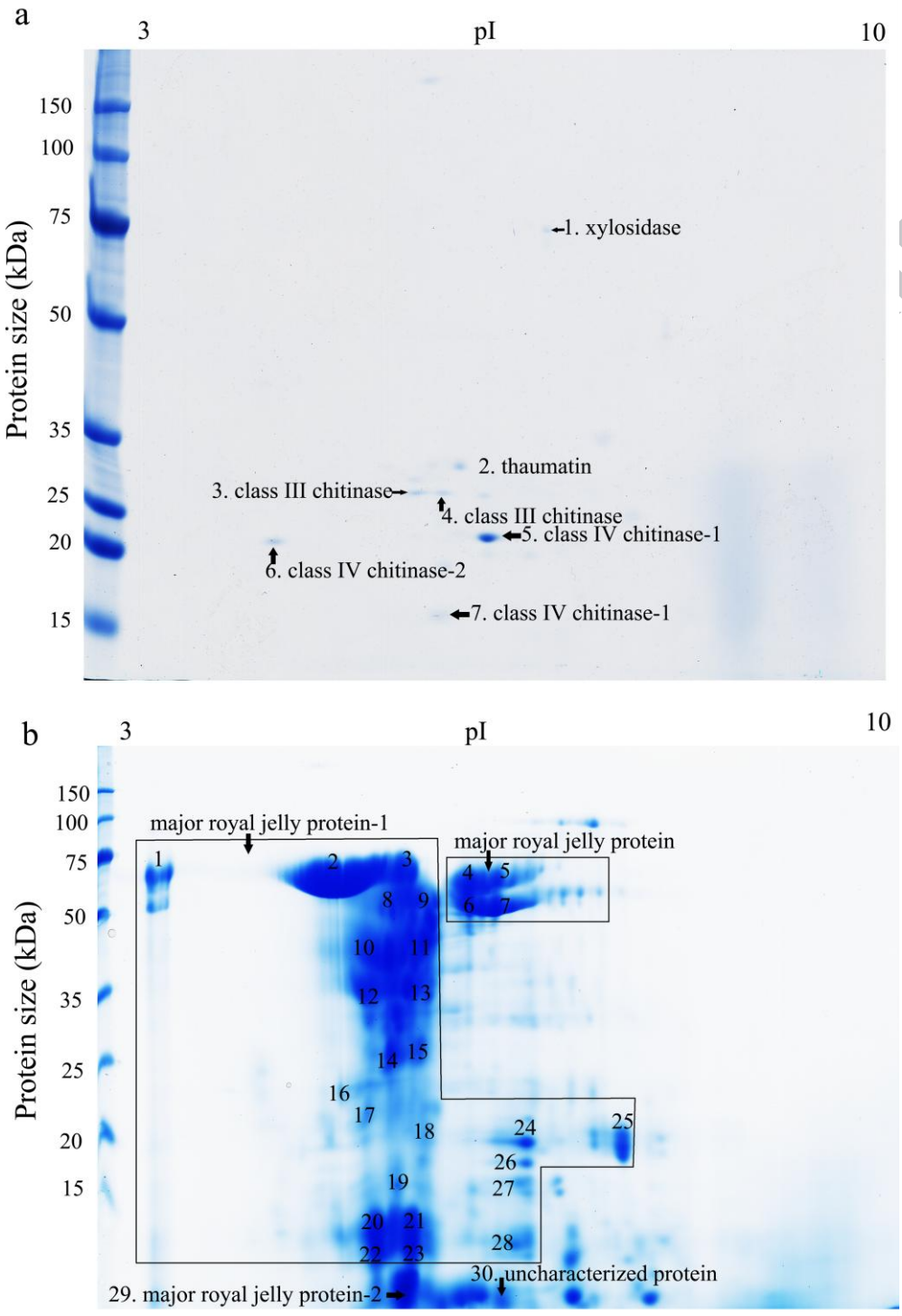
Fig. 3 Coomassie Brilliant Blue-stained two-dimensional (2D) gels of protein extracts from loquat floral nectar (a) and loquat honey (b). Identified proteins using MALDI-TOF/TOF were numbered and tagged on the gels.

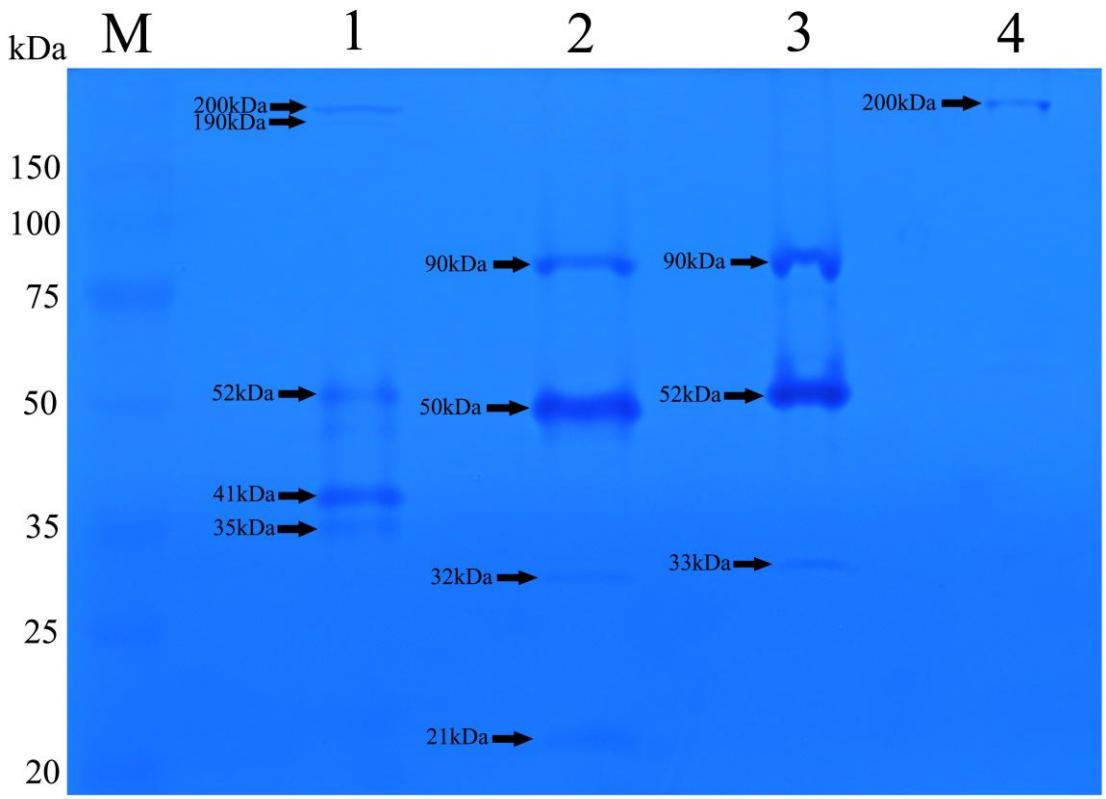
Fig. 4 Zymography of chitinase. Lane 1, rapeseed honey; lane 2, loquat honey; lane 3, loquat floral nectar; lane 4, *Camellia sinensis* floral nectar. The relative mobility for each band was indicated.

Fig. S1 Loquat pollen in loquat honey sample. Scale: 10 μm.









Highlights

- Bee-originated major royal jelly proteins are predominant proteinaceous components in loquat honey.
- Loquat nectar proteome mainly consisted of xylosidase, thaumatin, and two kinds of chitinases which were not detected in loquat honey using gel-based proteomic techniques.
- The zymography and content of nectar-originated chitinases is a potential marker for honey botanical origin authentication.